SURVIVAL OF BACTERIA UPON REPEATED FREEZING AND THAWING[1]

ARTHUR P. HARRISON, JR.

Department of Biology, Vanderbilt University, Nashville, Tennessee

Received for publication June 9, 1955

The purpose of the research presented in this paper has been to determine the effect of certain factors upon survival of bacteria after successive freezings. These factors include length of storage between each successive freezing, initial cell concentration, suspending medium, and the effect of aeration. No critical study of these factors appears in the literature.

MATERIALS AND METHODS

The following bacteria were employed as test organisms: *Lactobacillus fermenti* strain 69L-3, *Escherichia coli* strain 69L-15, and *Serratia marcescens* strain ATCC 274. They were cultivated as outlined previously (Major *et al.*, 1955).

Broth cultures of the organisms were centrifuged, the spent broth pipetted off, the cells resuspended in a minimal amount of fresh broth, and then the suspension diluted with additional broth to the desired cell concentration. The broth used as the suspending medium had the following percentage composition: yeast extract (B.B.L., dehydrated), 1.0; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.01; and "tween 80" (Atlas Powder Co.), 0.1. In some experiments with *L. fermenti* the suspending medium contained 1 per cent trypticase (B.B.L., dehydrated) and 2 per cent glucose as additional ingredients. In experiments where water was used as the suspending medium the cells from the centrifuged culture were resuspended in an equal volume of distilled water and again centrifuged, the supernatant pipetted off, the cells resuspended in a minimal amount of distilled water, and then the suspension diluted with additional water to the desired cell concentration.

The suspension was dispensed in 5-ml aliquots into test tubes (15 mm by 150 mm) fitted loosely with aluminum caps. Freezing was accomplished by immersion of the tubes in an alcohol bath at −22 C. After the contents of the tubes had solidified, which required about 5 min, the tubes were removed from the alcohol bath but kept within the cold chest at −22 C. Thawing was accomplished by quickly transferring a tube from the cold chest to a 35 C water bath and required from 2 to 3 min.

To facilitate discussion of the protocol employed, the experiments may be divided into three types. In the first, a batch of tubes were frozen and stored at −22 C as outlined above. Periodically, one tube was thawed and a 1-ml aliquot diluted and plated; this allowed the collection of data for the construction of a survival curve resulting from a single freezing-thawing. (One tube was used for one plating only; after removal of the 1-ml aliquot the tube was discarded.) Concurrently, after various storage intervals, a set of three to five tubes was thawed, frozen a second time and again stored at −22 C. Periodically, one tube was thawed and a 1-ml aliquot diluted and plated; this then permitted the construction of "secondary" survival curves. These curves, of course, have their points of origin on the primary survival curve and will be called "pendent" curves. Two such series of curves are plotted in figure 1 where the curve representing survival after a single freezing-thawing is heavily drawn and the pendent curves are drawn more lightly. In all figures, the point at 0 weeks represents the initial cell count of the test suspension, that is, the cell count per ml immediately prior to the first freezing.

In the second type of experiment, there may be three or four pendent curves, each having its origin at a point on the preceding curve. Such a series of curves represents survival after a number of alternate freezings and thawings (figures 2 and 3).

In the third type of experiment, the storage interval between successive freezings is too short to permit storage counts to be made. Experiments of this sort are simpler, since a single tube may be repeatedly frozen and thawed if 0.1-ml aliquots are removed for the platings, thereby not changing appreciably the volume of the test suspension

[1] This research was supported in part by funds provided by the Vanderbilt University Research Council.

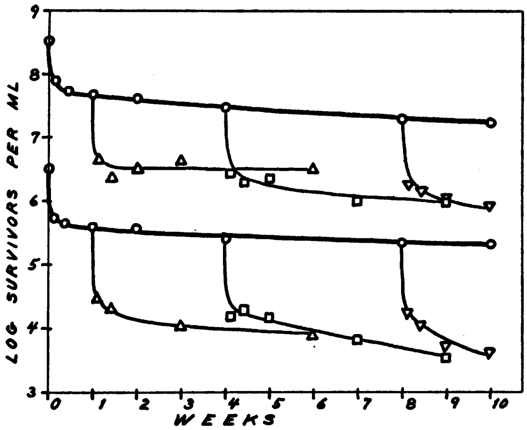

Figure 1. Survival of *Lactobacillus fermenti* in broth to a single freezing and to a second freezing after storage intervals of 1 week, 4 weeks, and 8 weeks.

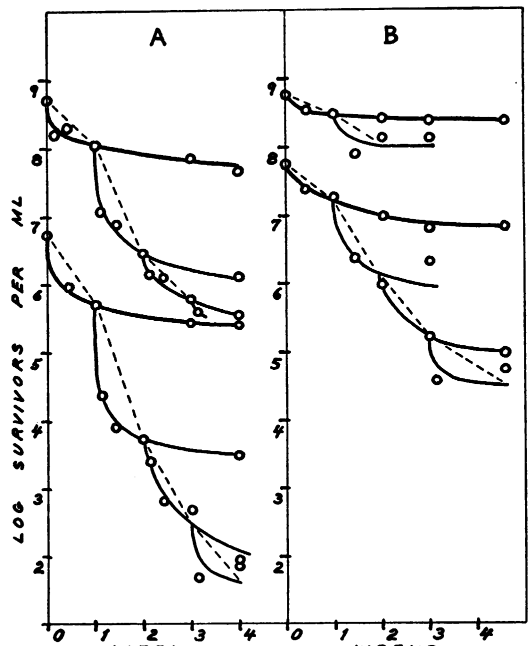

Figure 2. Survival of bacteria to successive freezings after storage intervals of 1 week. *A*, *Lactobacillus fermenti* in broth. *B*, *Escherichia coli* in water.

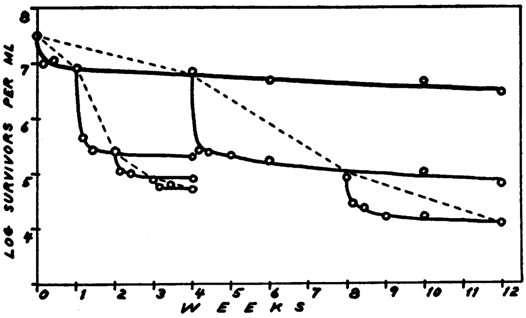

Figure 3. Survival of *Lactobacillus fermenti* in broth to successive freezings at storage intervals of 1 week and 4 weeks.

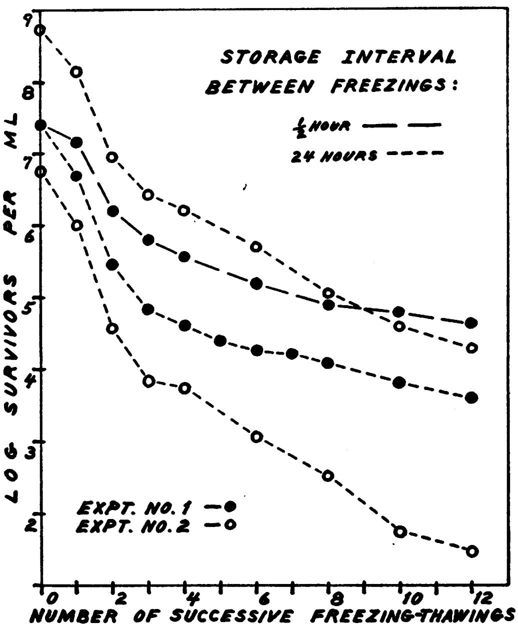

Figure 4. Survival of *Lactobacillus fermenti* at different initial cell concentrations in broth to successive freezings after storage intervals of ½ hour and 24 hours.

within the tube during the course of the experiment. Curves from experiments of this type are seen in figures 4 and 5, where the counts after each successive freezing-thawing are connected by a broken line as an aid in showing the relationship of the points to one another. In like manner, the counts resulting after each successive freezing-thawing in the case of experiments of the second type are also connected by broken lines.

Before plating or refreezing, the thawed suspensions were always gently agitated to ensure homogeneity. Dilutions for plating were prepared by transferring the 1.0- or the 0.1-ml aliquot to 99 ml of diluent in a dilution bottle.

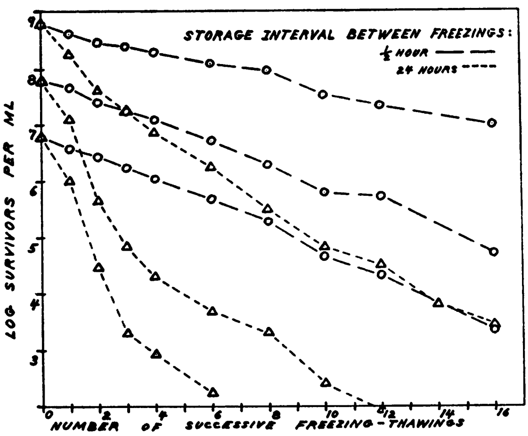

Figure 5. Survival of *Escherichia coli* at three different initial cell concentrations in broth to successive freezings after storage intervals of ½ hour and 24 hours.

Serial dilutions were made using 99 ml of diluent. The diluent contained 0.5 g of trypticase (B.B.L., dehydrated) and 0.6 ml of buffer solution (2.8 g KH_2PO_4, and 13.7 g K_2HPO_4, per 200 ml of water) per liter of distilled water. Plating was carried out as already outlined (Major *et al.*, 1955).

RESULTS AND DISCUSSION

It will be noted in figure 1 that in the case of a single freezing-thawing (the two heavily drawn curves), *L. fermenti* manifests a percentage survival which is constant and independent of the initial cell concentration. However, when the suspensions are subjected to a second freezing-thawing it becomes apparent that the initial cell concentration does, after all, exert an effect upon the suspensions, for careful examination shows that the percentage death represented by the three lower pendent curves is in every case somewhat greater than the percentage death represented by the three corresponding curves directly above. The effect of the cell concentration upon survival becomes more noticeable with yet additional successive freezing-thawings, as may be seen in figure 2*A* where *L. fermenti* at two initial cell concentrations has been successively frozen and thawed four times.

It is probable that the percentage survival of all bacteria is dependent upon the initial cell concentration. The difference in bacteria in this regard is whether a single freezing-thawing or repeated freezing-thawings are required to demonstrate this dependence.

In the case of a single freezing-thawing with *E. coli* the percentage survival varies in proportion to the initial cell concentration. The survivals resulting from a second freezing are somewhat erratic, but in general show the same behavior as in the case of *L. fermenti*. The dependence of survival upon the initial cell concentration may be seen also when washed cells of *E. coli* have been suspended in water and subjected to successive freezing-thawings (figure 2*B*).

With water as the suspending medium and at initial cell counts much below 10^7 per ml the storage counts of *E. coli* are unexpectedly low and erratic. Upon extensive dilution of a water suspension of *E. coli* some character of the suspension is so altered that the cells become unusually sensitive to freezing-thawing and it is not possible to plot smooth survival curves. For this reason the data obtained in this experiment at the lower initial cell concentrations have not been included in figure 2*B*. The phenomenon has been noted in replicate experiments and at about the same degree of dilution. This observation explains why contradictory conclusions regarding the comparative merit of broth and water as suspending media occur in the literature. Water may prove superior or inferior to broth as menstruum, depending upon the initial cell concentration and perhaps also upon the degree of washing of the cells prior to their final suspension and freezing.

The death resulting from each successive freezing is not of the same magnitude; with *L. fermenti*, the second freezing is always more lethal than either the first freezing or any of the subsequent freezings. This is true whether the length of storage between successive freezings be at intervals of 4 weeks (figure 3), 1 week (figures 2*A* and 3), 24 hours, or ½ hour (figure 4). In all cases, after the first three or four successive freezings, additional freezings cause relatively little death. With *E. coli*, when the interval between successive freezings is reduced to ½ hour, each successive freezing causes the same degree of death (figure 5), and the broken curve connecting the counts after each freezing-thawing may now be considered a straight line. At storage intervals of 1 day and greater, however, this organism behaves more like *L. fermenti*, at least

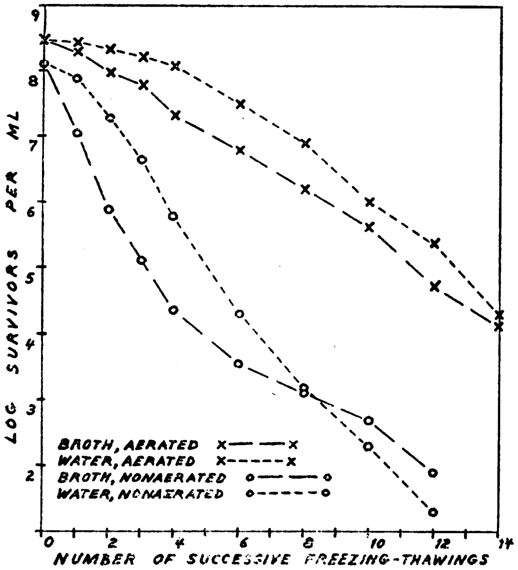

Figure 6. Comparison of the survival of aerated and nonaerated cells of *Escherichia coli* in broth and in water to successive freezings after storage intervals of 24 hours.

at the lower cell concentrations (figure 5). It is admitted that the difference in the death occurring after the first and after the second freezing would be expected if the cells were clumped. Indeed, this may be a factor, as it is difficult to completely disperse all clumped cells even with agitation and incorporation of "tween 80" in the medium. However, another factor may possibly be involved, because the amount of clumping observed microscopically in the suspensions prior to freezing is negligible, and because diluting the suspension prior to freezing and lengthening the storage interval between successive freezings (with *E. coli*) increases the bend in the curve (figure 5). With *Serratia marcescens*, where absolutely no clumping was observed, the results are the same; the curve is sigmoidal when the storage interval is 1 day, but approaches a straight line when the storage interval is reduced to ½ hour.

Hollander and Nell (1954) alternately froze and thawed suspensions of *E. coli* and *Diplococcus pneumoniae* and made platings after one, two, four, and eight such freezing-thawings. There was no storage interval between each successive freezing. They also observed that plotting logarithms of survivors versus the number of successive freezing-thawings resulted in a linear relationship.

It has already been reported from this laboratory (Major *et al.*, 1955) that aerated cells of such bacteria as *E. coli* and *S. marcescens* are more resistant to the lethal effect of a single freezing-thawing than are nonaerated cells. In the present study experiments were undertaken to compare survival of aerated and nonaerated cells of *E. coli* after repeated freezing and thawing; the results are plotted in figure 6. With water as menstruum, survival of aerated cells is far superior to that of nonaerated cells during the first six successive freezings, but with additional freezings this superiority is less pronounced. Likewise, aerated cells in broth are more resistant for the first six successive freezings; after additional freezings, however, the difference is less pronounced. In this figure a comparison may also be made between survival in broth and in distilled water. During the first few successive freezings survival is better in water. The difference is less pronounced after additional successive freezings, and eventually survival appears better in broth.

SUMMARY

Although *Lactobacillus fermenti* upon a single freezing-thawing manifests a survival which is constant and independent of the initial cell concentration, the percentage survival which results after a series of repeated freezings and thawings varies in proportion to the initial cell concentration.

With *L. fermenti*, regardless of the length of the storage interval between successive freezings, the lethal effect of the second freezing is greater than the lethal effect of any subsequent freezing or of the first freezing. Therefore, when logarithms of survivors are plotted versus the number of successive freezings a sigmoidal curve is obtained. The configuration of the sigmoidal curve is influenced by the length of the storage interval between the successive freezings and also by the menstruum, the initial cell concentration, and conditions under which the test organism is cultivated. With *Escherichia coli* and *Serratia marcescens* the curve may also be sigmoidal, except when the storage interval is short; then the curve becomes a straight line.

At moderately high cell concentrations, survival of *E. coli* to successive freezings is, at first, greater in water than in broth, but after additional successive freezings the converse may be true. Aerated cells of *E. coli*, whether suspended in water or broth, at first survive much better than nonaerated cells, but upon additional freezings the difference becomes less pronounced.

REFERENCES

HOLLANDER, D. H. AND NELL, E. E. 1954 Improved preservation of *Treponema pallidium* and other bacteria by freezing with glycerol. Appl. Microbiol., **2**, 164–170.

MAJOR, C. P., McDOUGAL, J. D., AND HARRISON, A. P., JR. 1955 The effect of the initial cell concentration upon survival of bacteria at −22 C. J. Bacteriol., **69**, 244–249.